Whole genome re-sequencing of non-model organisms: lessons from unmapped reads

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Abstract

Unmapped reads are often discarded from the analysis of whole genome re-sequencing, but new biological information and insights can be uncovered through their analysis. In this paper, we investigate unmapped reads from the re-sequencing data of thirty-three pea aphid genomes from individuals specialized on different host plants. The unmapped reads for each individual were retrieved following mapping to the *Acyrthosiphon pisum* reference genome and its mitochondrial and symbiont genomes. These sets of unmapped reads were then cross-compared, revealing that a significant number of these unmapped sequences were conserved across individuals. Interestingly, sequences were most commonly shared between individuals adapted to the same host plant, suggesting that these sequences may contribute to the divergence between host plant specialized biotypes. Analysis of the contigs obtained from assembling the unmapped reads pooled by biotype allowed us to recover some divergent genomic regions previously excluded from analysis and to discover putative novel sequences of *A. pisum* and its symbionts. In conclusion, this study emphasizes the interest of the unmapped component of re-sequencing datasets, and the potential loss of important information. We here propose strategies to aid the capture and interpretation of this information.

Keywords: NGS - unmapped reads - comparative genomics – aphids - symbionts – adaptive divergence

1 Introduction

Next Generation Sequencing (NGS) and whole genome re-sequencing is nowadays commonly used to identify genomic variants that underlie phenotypic variations, genetic diseases, adaptation or speciation in natural populations. Typically, the reads are mapped against a reference genome and the genotypes (i.e. SNP and structural variant calls) are based on these mapped reads (Altshuler et al, 2010; Nielsen et al, 2011). In addition to universal caveats regarding unknown insertions and/or genomic contamination, which can be overlooked in pure mapping approaches, non-model organisms may suffer from the poor quality of the nuclear reference genome and incomplete symbiont or organellar genomes. Moreover, mapping is constrained by the level of divergence between the reads and the available reference sequence (Sousa & Hey 2013). The resulting ascertainment bias could be problematic, especially when studying adaptation or speciation processes, as genomic regions of interest are expected to display important levels of divergence. These different issues produce a non-negligible fraction of unmapped reads, whose sequences are generally disregarded in favor of the mapped reads in the subsequent steps of the analysis, despite potentially containing useful information. This study offers one strategy for mining the unmapped reads in order to extract relevant biological knowledge, leading to advice and recommendations for other re-sequencing projects.

We investigated this question in the context of a large scale re-sequencing project on the pea aphid species complex. The pea aphid *Acyrthosiphon pisum* is a phytophagous insect which feeds on host plants in more than 20 Fabaceae genera. This species forms a complex of sympatric populations, or biotypes, each specialized on one or a few legume species (Simon et al, 2003; Via, 1991). Peccoud et al (2009a) showed that these biotypes include at least eight partially reproductively isolated host races and three cryptic species, forming a gradient of specialization and differentiation potentially through ecological speciation. This complex of biotypes started to diverge between 8,000 and 16,000 years ago, with a burst of diversification at an estimated 3,600–9,500 years (Peccoud et al. 2009b). In addition, the pea aphid is associated with an obligatory endosymbiont, *Buchnera aphidicola*, which is found in specialized cells called bacteriocytes and provides its host with essential amino acids. The pea aphid also harbors several facultative symbionts whose distribution is strongly correlated with plant specialization of their hosts (Ferrari et al, 2012; Henry et al, 2013, Simon et al, 2003) and it has been posited that some of these symbionts could play a role in plant adaptation, although clear evidence is still lacking (McLean et al, 2011; Tsuchida et al, 2004).

This study was carried out on thirty-three aphid re-sequenced genomes from 11 different plant-adapted biotypes. The reads were mapped against the *A.*

pisum reference genome, its mitochondrial genome and its known obligate (*B. aphidicola*) and facultative symbiont genomes. The *A. pisum* genome (530 Mb) was assembled using a combination of sequencing technologies (International Aphid Genomics Consortium, 2010; www.aphidbase.com). Although a second version of the *A. pisum* reference genome has since been released (International Aphid Genomics Consortium, 2010), the genome assembly remains highly fragmented (23,924 scaffolds) and it has not been subjected to the same level of scrutiny and finishing as the genomes of model organisms such as *Drosophila*. Moreover, symbiont genome sequences may not be well characterized for this species, and genomic divergence is expected to be important within the whole complex. As a result, a sizeable portion of the reads was not mapped.

In this paper, we scrutinized these unmapped reads by performing cross-comparisons between the sets, assembling the reads by biotype and analyzing the resulting contigs. We used tools developed for NGS such as *ABySS* (Simpson et al, 2009) and *Compareads* (Maillet et al, 2012), and more classical ones such as the BLAST suite of tools (Altschul et al, 1990). This analysis revealed that meaningful biological information is contained in the unmapped reads and could help to recover some divergent genomic regions previously excluded from analyses and to discover putative novel sequences of *A. pisum* and its symbionts.

2 Material and Methods

2.1 NGS data

Thirty-three pea aphid genomes were paired-end re-sequenced using the Illumina HiSeq 2000 instrument with around 15x coverage for each genome. The individuals belonged to different populations each referred to as a biotype due to their adaptation to a specific host plant. In this study, eleven biotypes were each represented by three individuals (Table S1 in Supplementary Material). Reads were 100bp long, sequenced in pairs with a mean insert size of 250 bp and between 32.5 and 59.2 million read pairs (42.5 million on average) were obtained for each individual (see Supplementary Material).

Reads were mapped using *Bowtie2* (Langmead and Salzberg, 2012) with default parameters (up to 10 mismatches per read, or fewer if indels are present – command-line in Supplementary Material) to a set of reference genomes. We also tested another popular mapper, BWA (Li and Durbin, 2009), but the percentage of unmapped reads was higher than for *Bowtie2* (on average over the 33 individuals, 6.1% vs. 3.7% for BWA and *Bowtie2*, respectively). The reference set comprised the published pea aphid *A. pisum* reference genome (IAGC, 2010), and its mitochondrial genome along with the genome of its primary bacterial symbiont and several secondary symbiont genomes reported for the pea aphid (*Hamiltonella defensa*, PAXS or X-type*, Regiella insecticola*, *Rickettsia* sp., *Rickettsiella* sp., *Serratia symbiotica*, *Spiroplasma* sp., *Wolbachia* sp., Oliver et al, 2010; Russell et al, 2013). When available we took the reference genome sequence of the symbiont associated with the pea aphid (i.e0 *Hamiltonella defensa 5AT* (CP001277.1)*, Regiella insecticola R5.15* (AGCA00000000.1)*, Serratia symbiotica* str. Tucson (AENX00000000.1))*,* otherwise genomes of the closest symbionts were used as reference (i.e. *Rickettsia* sp. endosymbiont of *Ixodes scapularis* (NZ_CM000770.1), *Rickettsiella grylli* (AAQJ00000000.2), *Spiroplasma melliferum* KC3 (AGBZ00000000.1) and *Wolbachia* sp. strain wRi (CP001391.1)). Note that we could not map reads to PAXS sequences because no genome is currently available for this symbiont either for *A. pisum* or other host organisms. Various statistics about the quality of the mapping were recorded and we calculated for each individual the average coverage for each reference genome used.

2.2 Extraction of unmapped reads

Fragments for which both reads of the pair did not map to the reference genomes were extracted from the BAM file (mapping result file) using *Samtools* features (Handsaker et al, 2011). In order to check the quality of the unmapped reads, *Prinseq* (Schmieder and Edwards, 2011) was used. Sequences were trimmed if, working from the 3' end of the read, base quality dropped below 20 within a window of 10 nucleotides. Read pair information was not preserved and only sequences of at least 66 nucleotides in length were retained for the analysis. Quality-trimmed single-end unmapped read sets were used as the input to the pipeline.

2.3 Pipeline for the analysis of unmapped reads

The analysis pipeline, shown in Figure 1, was composed of three major stages: i) pairwise comparisons between unmapped read sets, ii) *de novo* assembly of pooled sets of reads and iii) analysis of the assembled contigs. Pairwise comparisons between the read sets were computed in order to identify biologically relevant signals and to define groups of individuals based on the quantity of similar reads. The second part consisted of the assembly of the common reads within previously defined groups. The contigs larger than 1 kb were then analyzed in terms of size, read coverage and similarity to reference genomes.

Figure 1:

2.3.1 Comparison of unmapped reads

Compareads (Maillet et al, 2012) was used to compare the read content of the trimmed unmapped read set in each individual in a pairwise manner: this software can find similar reads between two sets of reads in an assembly-free manner. To be considered a match, a read of set A needs to share at least 2 non-overlapping kmers of size 33 with at least one read of set B. This comparison thus gives two percentages of similarity between sets A and B: the percentage of reads of A similar to reads of B and vice versa. For all pairwise comparisons, a symmetric similarity score was also provided, computed as follows: $\frac{AinterB+BinterA}{N_A+N_B}$, with *AinterB* the number of reads in set A similar to reads in set B, *BinterA* the number of reads in set B similar to reads in set A, N_A and N_B the total number of reads in sets A and B respectively.

The 33 samples were classified based on this similarity measure, using *R* (version 2.15) software with the maximum distance for the distance matrix and the complete linkage method for hierarchical clustering (function heatmap.2 from gplots package).

2.3.2 Assembly

We pooled common unmapped reads from the three individuals that belonged to the same biotype, i.e. reads present in at least one pairwise comparison between individuals of a biotype were all concatenated in one fastq file. The *de novo* assembler *ABySS* (Simpson et al, 2009) was used to assemble these common unmapped reads for each biotype. The size of the k-mer for the De Bruijn graph was set to 31.

To calculate the contig coverage statistics, the sets of unmapped reads were re-mapped to the obtained contig sequences using *Bowtie2* (default parameters) and the number of mapped reads was obtained using *Samtools*. In accordance with the mean coverage observed in the main dataset (i.e. where the pea aphid nuclear genome had an average coverage of 15x in each individual), and since reads from two to three individuals were pooled at this step, we considered that contigs with coverage ranging from 20x to 60x likely issued from the pea aphid (*nuclear-like* coverage), whereas contigs with higher coverage were considered more likely to derive from the symbionts (*symbiont-like* coverage) or repetitive sequences.

2.3.3 Comparison and analyses of contigs

BLASTClust was used to assess whether large homologous contigs (longer that 1 kb) could be found in different biotypes. A match was retained between two sequences if they were 80% identical over at least 90% of each sequence length. The contigs were then assayed by BLASTn search against the pea aphid reference genomes (nuclear, mitochondrion and symbionts) in order to ascertain their origin. Contigs with hits with an e-value less than 1e-50 were considered to represent highly divergent region of the *A. pisum* or its known symbiont genomes, i.e. assumed to contain reads that could not be mapped during the first mapping step.

2.4 *De novo* assembly and characterization of an aphid symbiont genome

We performed the following analyses to assemble the genome of a bacterial symbiont detected in the unmapped read set of the individual Vc3. First, starting from the full read set of Vc3 (40.3 million read pairs), reads were filtered according to their k-mer coverage to obtain only the reads originating from the targeted genome and thus avoid simultaneously assembling the whole nuclear pea aphid genome. Given that the targeted genome had an average read depth in Vc3 of around 600x, only reads for which 68% of the length was covered by 31-mers present at least 100 times in the dataset were retained, using *readFilter* (P. Peterlongo et al. unpublished) a custom software based on k-mer counts performed by the *DSK* software (Rizk et al, 2013). Reads that could be mapped to the *B. aphidicola* or mitochondrial genomes were removed since their coverage levels would otherwise lead them to be retained in this read set. Only read pairs that remained intact following these filtering steps were included, and these pairs (which totaled 8.8 million read pairs) were assembled using *SPAdes* (Bankevich et al, 2012), which has been reported to perform well with bacterial genomes (Magoc et al, 2013). Several k-mer sizes were combined in *SPAdes* (31, 41, 63, 81, 89), with default values employed for the other parameters. We kept contigs longer than 500 bp and removed those aligning with the non-*Spiroplasma* reference genomes. Alignments were performed with the global aligner *Mummer* (Kurtz et al, 2004). We used *GeneMarkS+* (Besemer *et al*, 2001) to predict proteins in the remaining contigs. These proteins were then compared to the NR database (version 22/01/2014) using BLASTp.

2.5 Identification and analysis of potentially divergent regions of the reference genome

To delineate potentially divergent regions of the reference genome which were present in the most divergent biotype (*L. pratensis*), contigs obtained from the unmapped reads of this biotype were aligned against the nuclear pea aphid genome with *Mummer*. The regions matching the reference genome with more than 80% identity and larger than 500bp were retained for further analyses.

In these regions, several metrics were computed, including the read depth at multiple mapping stringencies and SNP calling statistics. Read depth was computed first from the initial mapping obtained with Bowtie2 and also following mapping with *Stampy* (Lunter and Goodson, 2011), an aligner which is reported to perform well when mapping to a divergent reference. SNP calling statistics were collated from the results of the *GATK* (DePristo et al, 2011) pipeline applied to the complete dataset of 33 genomes. This pipeline consisted of PCR duplicate removal, indel realignment, base quality recalibration, and genotyping with the UnifiedGenotyper. We used the number of “undefined” calls, i.e. polymorphic positions in the genome for which the genotype could not be determined by UnifiedGenotyper, as a proxy for alignment success. Finally, the gene content of these regions has been established using the version 2.1 of the official gene set of the pea aphid provided by AphidBase (Legeai et al, 2010).

3 Results

3.1 Mapping to reference genomes confirms variation in symbiotic composition between individual host genomes

The coverage of the *A. pisum* nuclear genome was 14.3x on average (min=10.6x and max=19.96x) while its mitochondrial genome was covered 946.0x on average (min=257.09 and max=3245.60x) and its obligate symbiont genome, 748.8x on average (min=138.08x and max=1509.03x). The coverage of the facultative symbiont genomes depended strongly on the individual host and varied from 0x to 117.7x. Observed variation for symbiont genome coverage among pea aphids was strongly linked to the infection status of the hosts. Indeed, when we compared expected symbiotic composition based on PCR detection tests and results of mapping, we obtained a good match in most cases: the presence of a given symbiont as detected by diagnostic PCR was confirmed by >2x coverage of reads that mapped against the reference genome (Table S2 in supplementary material). There were however several exceptions to this pattern, namely *Ricketssia*, *Rickettsiella* and *Spiroplasma* symbionts for which genomes from a pea aphid host are currently not available. It should be noted that positive individuals for each of these three symbionts showed a weak but detectable number of reads that mapped against the closest reference genome found in databases (i.e. Ps1, Ml1 and Ml3 individuals infected by *Rickettsia*, Tp3, Vc1 and Vc3 individuals infected by *Spiroplasma* and Ms1 individual infected by *Rickettsiella* in Table S2). Note also that no reads mapped to the *Wolbachia* genome, confirming the absence of this symbiont in our selection of *A. pisum* genotypes.

3.2 A non-negligible fraction of reads does not map

For a given individual, there were between 0.6 and 7 million pairs of reads (mean = 1.3 million) where both reads did not map to any of the reference genomes (nuclear genome, mitochondrion or known symbionts). This constituted an average of 3.7 % of the initial read sets. Moreover, most of these reads were of good quality, as shown in Figure 2, since few reads were removed (about 17 %) by quality trimming (see Methods).

Figure 2 :

A direct analysis of these read datasets did not allow to characterize the unmapped reads in comparison to the mapped ones, in terms of sequence complexity (Shannon entropy) or signal for repeats (no enrichment of sequence matches with small RNAs targeting pea aphid transposable elements). However, the small size of the reads makes such direct analyses difficult and limits the sensitivity of such characterization.

We can also see in Figure 2 that the fraction of unmapped reads varied between individuals. In particular, the individual Vc3 showed an atypically large amount of unmapped reads with more than 14 million reads representing 18.5% of the initial read set for this individual. For some biotypes, the fraction of unmapped reads was very similar across all individuals, perhaps implying a common cause of mapping failure. However the fraction of reads did not seem to be correlated with the divergence of the individuals (or biotypes) with respect to the reference genome. The absence of this correlation suggests that the failure to map is not a simple consequence of inappropriate mapping parameters, as if mapping were too stringent we would expect to obtain a correlation between the unmapped fraction and biotype divergence from the reference.

3.3 Unmapped reads contain biologically meaningful information

Each set of unmapped reads was compared to all other sets using *Compareads*. Across the 1056 (33x32) pairwise comparisons, the percentage of common reads between 2 individuals varied greatly, from 6 to 95% with an average value of 50% (see Figure S3 of Supplementary Material). For all but one individual, there was at least one other individual with which it shared 50% of its reads.

Interestingly there was a significant difference when comparing individuals of the same biotype, where on average 70% of reads were shared between individuals, versus comparisons between individuals of different biotypes, which on average shared 48% of reads (p-value $<10^{-16}$ for the Welch 2-sample test). This trend was confirmed by the hierarchical classification of individuals based on the pairwise similarity scores computed from the read set intersections (see Methods). Indeed, we can see in Figure 3 that individuals belonging to the same biotype were largely clustered together.

Figure 3:

One extreme case is the *L. pratensis* biotype, which is known to be the most divergent biotype and is considered a cryptic species (Peccoud et al, 2009a). It showed a very specific profile on the heatmap with strong similarity within this biotype (yellow group on Fig. 3): a *L. pratensis* individual shared on average 72 % of its unmapped reads with another *L. pratensis* individual, whereas only 23 % were shared with an individual of another biotype.

These results show that the sets of unmapped reads contain sequence information specific to biotype or group of individuals, and therefore may contain valuable sequences for biological analyses.

3.4 Where do these sequences come from?

In order to get longer and more readily interpretable sequences, we assembled them conjointly by biotype, using the assembler *ABySS*. Pools of unmapped read sets were used as inputs to obtain sufficient coverage for good quality assemblies. Since the individual classification accorded with the biotype composition and because individuals from the same biotype are genetically closer than those from other biotypes (Peccoud et al, 2009a), we pooled unmapped reads that were shared between at least 2 of the 3 individuals that belonged to the same biotype. By removing reads uniquely present in a single individual, putatively low coverage sequences were excluded, limiting one potential source of noise to the assembly process. Overall, 94 Mb of contig sequences, each ranging from 100 bp (shorter contigs were filtered) to 35.6 kb, were assembled. On average, 45 % of the unmapped reads could be remapped to the assembled contigs. The average N50 was low (around 428 bp), but we obtained more than 11 800 contigs larger than 1 kb (see Table 1).

The subsequent analysis considered contigs larger than 1kb in more detail. Coverage of the contigs varied considerably, with 57 % of them having a *nuclear-like* coverage, i.e. between 20 and 60x (see Material and Methods), consistent with an origin from the pea aphid nuclear genome. On the other hand, 14 % of contigs had coverage greater than 60x which would be consistent with an origin from bacterial symbionts, the mitochondrion, or repeated sequences. Contigs with coverage lower than 20x (29%) could correspond to sequences from other microbes (including unreported symbionts) that are in low abundance in the aphid host.

Table 1

Alignment of the contigs to the set of reference sequences can also suggest a potential genomic source. Overall, 63 % of the contigs had a significant blast hit to one of the reference genomes, with the large majority matching with the nuclear pea aphid genome (89 %).

As was found in the coverage analysis, the BLAST analysis revealed biotype-specific trends (Fig. 4). Both approaches can thus be applied to classify the contigs into one of the two main origins: either symbiotic or nuclear. Moreover, the attributions by coverage and BLAST are largely consistent, with a concordant origin for 93 % of the contigs with an origin assigned by both methods.

Figure 4:

3.4.1 Sequences of symbiotic origin

Three biotypes contained a sizeable proportion of sequences with a putative symbiotic origin: *P. sativum, V. cracca* and *M. lupulina*. Both the *P. sativum* and *M. lupulina* biotype contig sequences predominantly showed significant similarity to reference symbiont genomes (see Figure 4). In line with this symbiont status these contigs had a high coverage (more than 140x on average). The detected similarity was due to one particular symbiont genome: *Rickettsia* sp. endosymbiont of *Ixodes scapularis* (and included in the reference genome set). However, very few reads had mapped initially to this genome, which had an overall coverage of only 3x for the *P. sativum* and *M. lupulina* biotypes, and was absent from all other biotypes. This suggested that the chosen reference genome for *Rickettsia* was too distant from the actual pea aphid symbiont. By comparing these contigs to other *Rickettsia* species we identified *R. bellii* as a more closely related species. This closer relationship was also confirmed by a phylogenetic analysis of 16S ribosomal RNA genes from all available *Rickettsia* species having their complete genome sequenced (Fig. S1 in supplementary material). Substituting this genome as a reference resulted in an improved coverage for both *P. sativum* and *M. lupulina* biotypes and confirmed the presence of this facultative symbiont in individuals that showed negligible coverage when their reads were mapped to *R. ixodes* (Table S2 in supplementary material). However, the discrepancy between this coverage level and that observed in the assembled contigs, which was over 2-fold higher, suggests that *Rickettsia* from *A. pisum* may diverge significantly from *R. bellii* which requires to characterize its genome.

Unlike the *P. sativum* and *M. lupulina* biotypes, the *V. cracca* biotype contigs showed almost no similarity to the reference symbionts despite a coverage signal which averaged 602x and was thus consistent with symbiont origin. When aligning these contigs to the NR nucleic acid database, we found few matches and typically only low similarity scores but noted that these hits were enriched in sequences from the Mollicute group and, more precisely, the *Spiroplasma* genus. This implied that some individual genomes of the pea aphid contained a *Spiroplasma* symbiont whose genome is distant from available *Spiroplasma* genomes in the public databases. This hypothesis was confirmed by the fact that three genotypes (of which two *V. cracca*) of the pea aphid were positive for *Spiroplasma* infection based on PCR-specific detection (Table S2 in supplementary material). Therefore, *Spiroplasma* was likely present in at least three individuals, but in high abundance in only one: Vc3. Indeed, most of the *V. cracca* unmapped reads came from this single *V. cracca* individual, which had 5 times more unmapped reads than the average (more than 14 million reads, see Figure 2). Based on the hierarchical classification in Figure 3, Vc3 was grouped with the individuals Vc1 and Tp3 (in agreement with PCR results), with 90.5 and 95% of its unmapped reads being similar to these two individuals respectively. The high abundance of reads from this uncharacterized source in Vc3 led us to attempt the *de novo* assembly of this *Spiroplasma* genome. The assembly was performed with *SPAdes*, after having extracted only putative "*Spiroplasma*" reads from the full Vc3 read set (see Material and Methods). The final assembly contained 509 contigs longer than 500 bp (2442 bp on average), totaling 1.2 Mb of sequence. While at the nucleotide level, these contigs showed weak similarity to available *Spiroplasma* genomes, at the protein level their relationship with Mollicute (and mainly *Spiroplasma*) proteins was confirmed for 546 annotated genes (on contigs summing to 780 kb). Moreover, the assembly size, low GC content (24%) and the fragmented assembly were consistent with known *Spiroplasma* genome features: the genome size varies from 1.4 to 1.9 Mb, GC content is around 26 % and the genomes contain lots of repeated sequences and viral elements that make the assembly task harder (Carle et al, 2010; Lo et al, 2013). Additionally, a phylogenetic analysis of its 16S RNA gene confirmed its membership to the *Spiroplasma* genus and the absence of any close relative with a complete genome available in the databases (Fig. S2 in supplementary material).

Finally, when unmapped reads were re-mapped to the partially assembled genome of *Spiroplasma* isolated from *A. pisum*, individuals which had been found to be positive for *Spiroplasma* by a PCR-based diagnostic assay but which had a negligible coverage when their reads were mapped to *S. melliferum*, registered a high coverage on contigs from the *A. pisum*-derived *Spiroplasma* (up to 1185x for some individuals, Table S2, supplementary material). In one case, the sensitivity of the sequence analysis may have exceeded that of the PCR test since individual Lc3 was PCR-negative for *Spiroplasma* but recorded on re-mapping, suggesting a possible infection under the threshold of PCR detection.

3.4.2 Sequences of nuclear origin

All biotypes possessed contigs with a putative nuclear origin, as shown on Figure 4. Some of these contigs were similar between several biotypes or even between all biotypes. We clustered the contigs together using BlastClust and obtained overall 10.1 Mb of distinct sequences having a *nuclear-like* coverage, of which 4.2 Mb had no similarity to the reference genome of *A. pisum*. Some of these are likely to be insertion polymorphisms, whereas the 8.6 kb that are shared in at least 8 biotypes could represent pea aphid sequences missing from the current reference assembly either due to error or to deletions in the individual genome that was used to build the reference genome.

Aside from these common sequences, the *L. pratensis* biotype was particularly enriched in sequences with a putative nuclear origin (Figure 4). Most of its contig sequences had a significant blast hit to the nuclear reference genome (2.4Mb (69.8 %) of total contig length) and a nuclear-like coverage (86 % of total length), suggesting that these contigs were assembled from reads that were too divergent to map in the first place.

1137 regions (covering 1001 kb) that exhibit similarity to a *L. pratensis* contig over at least 500bp were then delimited on the reference genome, using the global aligner *Mummer*. The analysis of read coverage in these regions uncovered two types of region: “low-coverage” regions in which very few reads had mapped (coverage less than 30x for the three *L. pratensis* individuals combined, 377 regions summing to 337 kb), and “normal-to-high-coverage” regions (760 regions, 663 kb). While the latter could be explained by one or several divergent copies not present in the reference genome, the former are likely to be regions that are too divergent in all the *L. pratensis* genomes, in which we may miss important biological information. Indeed, we observed a high proportion of undefined SNP calls for *L. pratensis* samples in these “low-coverage” regions. On average each *L. pratensis* individual had 61% of undefined calls, whereas this percentage never exceeded 20% in these same regions for other biotypes (with an average of 13%). These are high values compared to the proportion of undefined calls over the whole genome (on average, 9% for *L. pratensis* samples and 3.7% for other biotypes). Moreover, half of the regions showed more than 50% of undefined calls for all 3 *L. pratensis* individuals. This supports the assertion that SNP information is lost because of unmapped reads.

When using a more sensitive mapping approach with *Stampy*, some of these missed SNPs could be recovered. For the three *L. pratensis* genomes, overall, 64% of the initially unmapped reads were re-mapped onto the set of reference genomes. Among these rescued reads, 0.66% mapped to the "low-coverage" regions which was more than expected knowing that these regions of interest represent only 0.06% of the whole genome. This sensitive mapping enabled recovery of, on average, 60% of undefined SNP calls, with 12.5% of regions completely resolved (i.e. with no undefined SNPs). However, for 54% of the regions, the total coverage (*Bowtie2* + *Stampy*) still did not reach normal levels and remained below 30x.

4 Discussion and conclusion

Whilst approaches for mining unmapped read sets for specific purposes have been described, for example for pathogen discovery (e.g. Kostic et al, 2011), this portion of reads is typically disregarded in re-sequencing projects. The sources of unmapped reads are various: they may derive from characterized or uncharacterized symbionts, bacterial, viral or eukaryotic pathogens, highly divergent genomic regions, genomic insertion sequences or library contaminants. The relative proportions of these contributions can vary, and factors such as reference genome quality and the genetic distance between reference and target can play a major role. Therefore, in non-model systems, both the contribution of unmapped reads to the dataset and the likelihood that these reads are a reservoir of useful biological information are increased. However, identifying the unmapped reads and ascribing them to specific sources is not trivial. We have here proposed a novel approach to rescue some of this potentially lost information and have explored the unmapped read sets in the context of thirty-three re-sequenced genomes from biotypes of the pea-aphid species complex.

The direct pairwise comparisons of read sets, before assembly, enabled the rapid identification of similar read sets and highlighted atypical samples and biotypes. Moreover, since the coverage of each individual alone was too low to expect a good quality assembly, merging samples in order to achieve sufficient coverage was necessary for *de novo* assembly quality. However, selecting and merging only reads common to a single biotype or population, would preclude the identification of other interesting sequences specific to one genotype or to a combination of individuals of different biotypes. Therefore a more in-depth analysis of the pairwise comparisons followed by the assembly of particular combinations of read sets could be interesting to conduct and may help to uncover unexpected links between individuals.

The assembly phase generated longer sequences than the pre-processed read sets, and these can be more efficiently analyzed and compared to sequence databases. However, although bacterial sequences, such as the ones obtained from *Rickettsia* and *Spiroplasma*, could be relatively easily assembled and led to large contigs, we observed that the remaining contigs were usually very short (N50 around 400 bp) and, probably one of the consequence is that a large fraction of the unmapped read sets could not be remapped on the shortest contigs. The recovery of short contigs may be influenced by our methods: extracting and assembling only unmapped read pairs would mean that we assemble only regions of high divergence which may be interspersed in the genome with less divergent regions that are well served by the mapping. In this case, we would predict that samples from the more divergent populations would have, on average, larger contigs of nuclear origin. This is supported in our case by the most distant biotype from the reference, *L. pratensis*, which shows the greatest proportion of large contigs with similarity with the nuclear genome and *de facto* the highest number of remapped reads (53.2%).

The final step of our approach was to align the contigs against the reference genomes (nuclear and symbionts) with less stringent similarity criteria than those used during the first mapping step of our process. This, together with the average read coverage of contigs, allowed us to ascribe a putative origin (nuclear or symbiont) of most of the larger contigs. For contigs of symbiont origin, this revealed notably the mis-specification of a reference genome and identified a closer representative species. Without this analysis, we would have concluded from the first mapping that this symbiont was absent (or at very low abundance) from all individuals. Moreover, this revealed the presence in three individuals of a symbiotic bacterium of the genus *Spiroplasma*, that has been previously reported for the pea aphid (Fukatsu et al, 2001) but never sequenced and for which we produced a first draft assembly of its genome. Again, the presence of this symbiont would have been completely missed with the first mapping.

In addition, this analysis allowed us to highlight specific parts of the nuclear genome which are enriched in the unmapped read set. These are large regions which are either absent from the reference genome, or show high divergence to the corresponding reference sequence such that each of the read pairs originating from it cannot be mapped. The latter explanation seems to be the most frequent in our dataset. This highlights the major drawback of classical comparative genomics approaches relying on a reference genome. The regions of the reference genome with important genomic divergence for some individuals will contain fewer mapped reads from these individuals and ultimately little divergence will be detected, leading to an erroneous interpretation. We confirmed this consequence of unmapped reads by observing an increased level of unassigned genotypes in these particular parts of the reference genome for the most divergent biotype.

This mapping issue could lead to the loss of valuable biological information or biases in the analysis of genomic variation. Careful calibration of mapping parameters to better handle sequence mismatches between reads and the reference genome can reduce the fraction of reads that cannot be mapped. We explored this by using the Stampy aligner to reprocess the unmapped reads, and could recover 64%. Whilst this offers an improvement on the original Bowtie mapping, most of the observed regions with missing genotype information remained unresolved and it is important to note that relaxing these settings will increase false positive mapping and also increases the time and computing resources required to process the datasets.

Here, our approach helped to recover those divergent regions and, having applied this strategy, the biological signals and functions of these regions can then be interrogated. In the case of the pea aphid dataset, the genic content of the regions will be investigated with a view to determining whether they are enriched in genes involved in host-plant adaptation (e.g. receptors and enzymes). More generally, recovery of these regions enabled them to be subjected to further study, for example to identify signatures of positive selection.

Supplementary information is available at Heredity's website.

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Table 1: Contig statistics. For each biotype, the number of unmapped reads in million (n reads) used for the assembly is indicated along with several statistics describing the properties for two contigs length cut-offs (100bp and 1kb), namely the number of obtained contigs (nb), their cumulative length (assbl. Mb), the percentage of reads (% reads) that could be mapped to the contigs and the N50 value.

Legends of figures

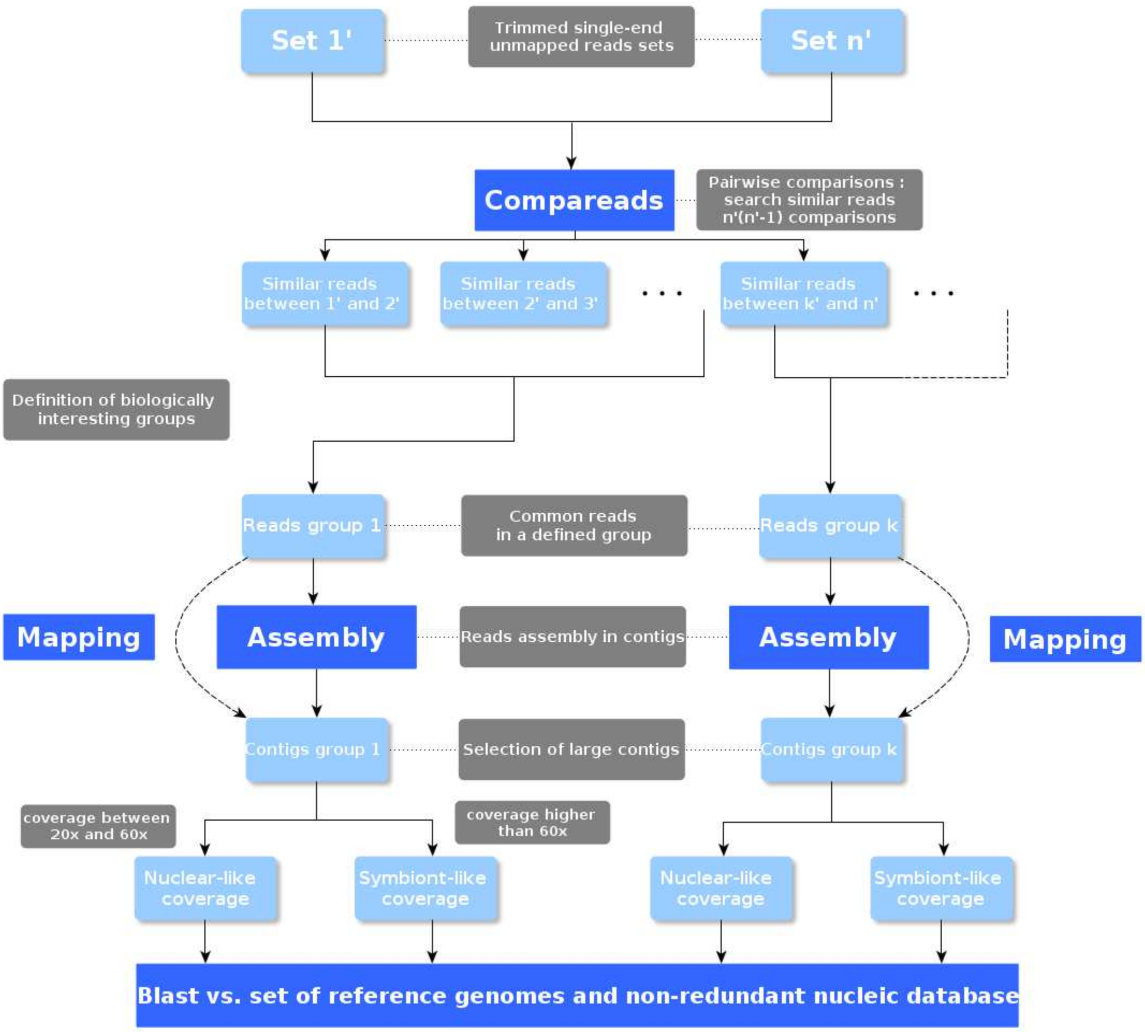
Figure 1: Global overview of the pipeline followed for the analysis of unmapped reads.

Figure 2: Percentage of unmapped reads (unmapped by pair) for each individual, after and before cleaning for quality. Individuals are grouped by biotype and sorted according to their known divergence with respect to the reference genome, the most divergent ones being at the right side of the figure.

Figure 3: Hierarchical classification of the sets of unmapped reads. Each color below the tree corresponds to a biotype. Colors in the heatmap are function of the similarity score between two samples, from low similarity in red to high similarity in yellow.

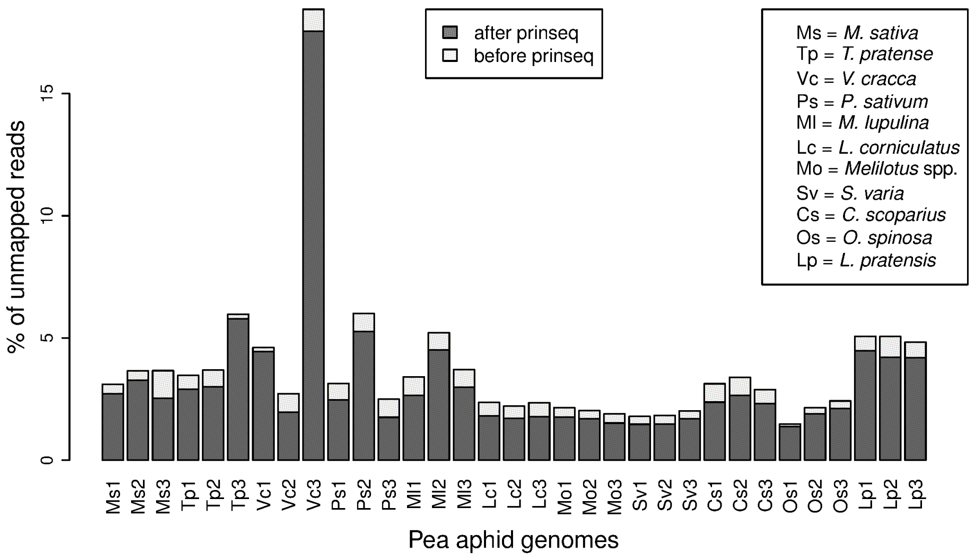
Figure 4: Analysis of contigs larger than 1kb in terms of blast matches and read coverage.

775



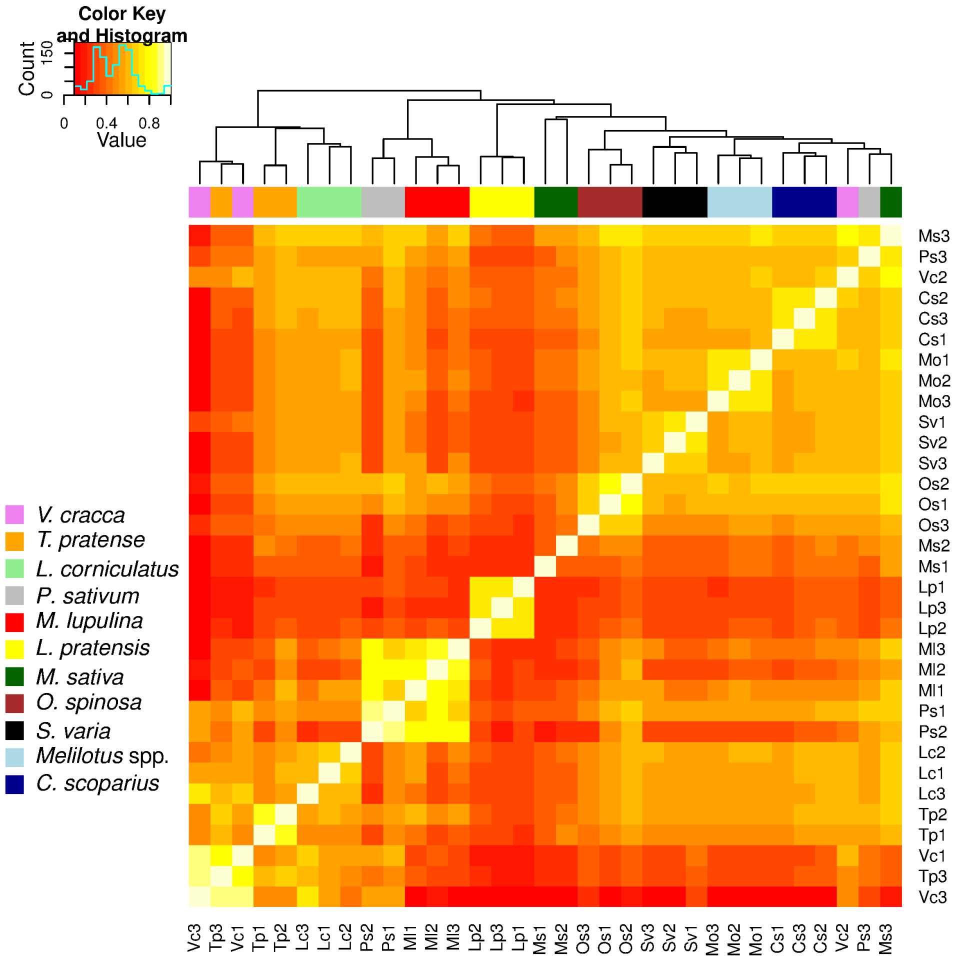
Set 1'
Trimmed single-end unmapped reads sets
Set n'
Compareads
Pairwise comparisons : search similar reads n'(n'-1) comparisons
Similar reads between 1' and 2'
Similar reads between 2' and 3'
Similar reads between k' and n'
Definition of biologically interesting groups
Reads group 1
Common reads in a defined group
Reads group k
Mapping
Assembly
Reads assembly in contigs
Assembly
Mapping
Contigs group 1
Selection of large contigs
Contigs group k
coverage between 20x and 60x
coverage higher than 60x
Nuclear-like coverage
Symbiont-like coverage
Nuclear-like coverage
Symbiont-like coverage
Blast vs. set of reference genomes and non-redundant nucleic database

Figure 1



after prinseq
before prinseq
Ms = M. sativa
Tp = T. pratense
Vc = V. cracca
Ps = P. sativum
Ml = M. lupulina
Lc = L. corniculatus
Mo = Melilotus spp.
Sv = S. varia
Cs = C. scoparius
Os = O. spinosa
Lp = L. pratensis
% of unmapped reads
0
5
10
15
Ms1
Ms2
Ms3
Tp1
Tp2
Tp3
Vc1
Vc2
Vc3
Ps1
Ps2
Ps3
Ml1
Ml2
Ml3
Lc1
Lc2
Lc3
Mo1
Mo2
Mo3
Sv1
Sv2
Sv3
Cs1
Cs2
Cs3
Os1
Os2
Os3
Lp1
Lp2
Lp3
Pea aphid genomes

Figure 2


Color Key
and Histogram
Count
0
150
0
0.4
0.8
Value
V. cracca
T. pratense
L. corniculatus
P. sativum
M. lupulina
L. pratensis
M. sativa
O. spinosa
S. varia
Melilotus spp.
C. scoparius
Ms3
Ps3
Vc2
Cs2
Cs3
Cs1
Mo1
Mo2
Mo3
Sv1
Sv2
Sv3
Os2
Os1
Os3
Ms2
Ms1
Lp1
Lp3
Lp2
Ml3
Ml2
Ml1
Ps1
Ps2
Lc2
Lc1
Lc3
Tp2
Tp1
Vc1
Tp3
Vc3
Vc3
Tp3
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Lc3
Lc1
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Ps1
Ml1
Ml2
Ml3
Lp2
Lp3
Lp1
Ms1
Ms2
Os3
Os1
Os2
Sv3
Sv2
Sv1
Mo3
Mo2
Mo1
Cs1
Cs3
Cs2
Vc2
Ps3
Ms3

Figure 3

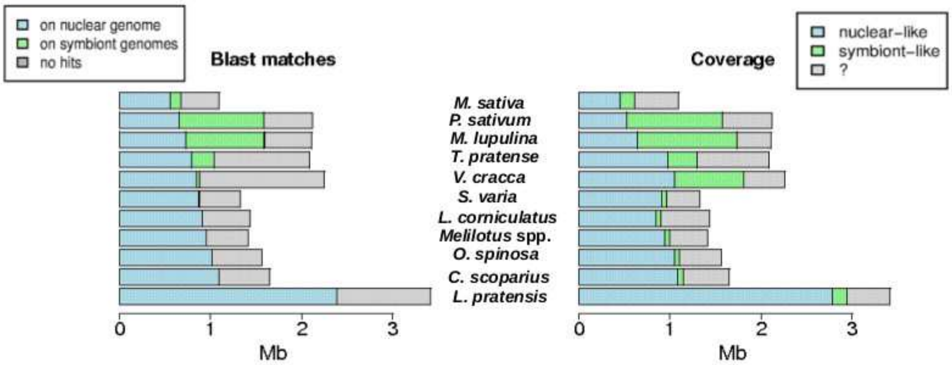
on nuclear genome
on symbiont genomes
no hits
Blast matches
Coverage
nuclear-like
symbiont-like
?
M. sativa
P. sativum
M. lupulina
T. pratense
V. cracca
S. varia
L. corniculatus
Melilotus spp.
O. spinosa
C. scoparius
L. pratensis
0
1
2
3
Mb
0
1
2
3
Mb

Figure 4